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Published in:
Applied Microbiology and Biotechnology

DOI:
10.1007/s002530051353

Publication date:
1998

Appl Microbiol Biotechnol (1998) 50: 697–703

ORIGINAL PAPER

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Screening and characterization of *Lactobacillus* strains producing large amounts of exopolysaccharides

Received: 20 March 1998 / Received revision: 12 August 1998 / Accepted: 12 August 1998

Abstract A total of 182 *Lactobacillus* strains were screened for production of extracellular polysaccharides (EPS) by a new method: growth in liquid media with high sugar concentrations. Sixty EPS-positive strains were identified; 17 strains produced more than 100 mg/l soluble EPS. Sucrose was an excellent substrate for abundant EPS synthesis. The ability to produce glucans appears to be widespread in the genus *Lactobacillus*. The monosaccharide composition of EPS produced by *Lactobacillus reuteri* strain LB 121 varied with the growth conditions (solid compared to liquid medium) and the sugar substrates (sucrose or raffinose) supplied in the medium. Strain LB 121 produced both a glucan and a fructan on sucrose, but only a fructan on raffinose. This is the first report of fructan production by a *Lactobacillus* species. EPS production increased with increasing sucrose concentrations and involved extracellular sucrase-type enzymes.

Introduction

Polysaccharides find numerous industrial applications (Roberts 1995; Sutherland 1993, 1998). The food industry is especially interested in natural thickeners, such as guar gum, locust bean gum, pectin, starch (all from plants), gelatin (animals), alginate, carrageen, agar (all from seaweed), xanthan gum and gellan gum (all from bacteria). Most of these are additives, however, and increasingly considered less desirable.

Lactic acid bacteria are food-grade organisms, possessing the GRAS (generally recognized as safe) status, and are known to produce extracellular polysaccharides (EPS), which contribute to the texture of fermented milk (Cerning 1990; Roller and Dea 1992). EPS from these bacteria thus may provide a new generation of food-grade thickeners. Lactic acid bacteria often also contribute positively to the taste, smell or preservation of the final product. These bacteria produce both homopolysaccharides (Cerning 1990; Dunican and Seeley 1965; Robyt 1995), e.g. glucans and fructans (*Leuconostoc mesenteroides*, streptococci), and heteropolysaccharides (van den Berg et al. 1995; Cerning 1990; Cerning et al. 1994; Grobben et al. 1995; Gruter et al. 1992; van Kranenburg et al. 1997; Stingele et al. 1996). The properties of EPS from lactic acid bacteria vary strongly, depending on monosaccharide composition, degree of branching, and types of glycosidic linkages present (Cerning 1990; Robyt 1995).

Synthesis of heteropolysaccharides by lactobacilli is currently being studied intensively (van den Berg et al. 1995; Cerning et al. 1994; Grobben et al. 1995; Yamamoto et al. 1994, 1995). Few studies have focused on the synthesis of homopolysaccharides by lactobacilli (Dunican and Seeley 1965; Pidoux et al. 1990; Sharpe et al. 1972). Limited information thus is available about glucan synthesis, and no reports have appeared about fructan synthesis by lactobacilli. Here we report the results of a new screening procedure for EPS-producing lactobacilli, using liquid growth media with high sugar concentrations. The effects of growth conditions on EPS production by two selected *Lactobacillus* strains were also studied.

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Materials and methods

Strains, media and batch fermentations

A wide variety of *Lactobacillus* species, originally isolated from diverse sources and habitats, e.g. (fermented) foods, the gastro intestinal tract of laboratory animals and human dental plaque, were used (TNO Nutrition and Food Research culture collection). *Lactobacillus reuteri* strains LB 121 and LB 180 have been deposited with the BCCM/LMG Culture Collection (accession numbers LMG 18388 and LMG 18389 respectively). All strains were grown anaerobically at 37 °C in MRS medium (de Man et al. 1960), solidified with 20 g/l agar when appropriate. EPS production was screened in modified MRS media containing 100 g/l glucose (MRS-g), fructose (MRS-f), maltose (MRS-m), raffinose (MRS-r), sucrose (MRS-s), galactose (MRS-gal) or lactose (MRS-l), instead of the 20 g/l glucose normally present in MRS medium. All media were autoclaved at 121 °C for 15 min. In the preparation of modified MRS, the sugars were autoclaved separately.

Infusion flasks (350 ml) equipped with a magnetic stirrer, incubated in an anaerobic glove cupboard, or Applikon (Schiedam, The Netherlands)/Bioflow III (New Brunswick Scientific, Edision, USA) fermentors (2.5 l), flushed with nitrogen, were used for anaerobic batch fermentations at 37 °C. In the latter case, *Lactobacillus* strains were grown at pH 4.8 or 5.8, controlled by automatic addition of 2 M or 4 M NaOH.

Each experiment described was performed at least in triplicate; data presented are averages with a standard deviation of less than 10%.

Screening for EPS synthesis

Modified MRS media (10 ml) were inoculated (1%) with strains pregrown in MRS medium. After 3 days of incubation, 1-ml culture samples were centrifuged (4 min at 11 000 *g*). Two volumes of cold (4 °C) ethanol were added to one volume of culture supernatants; the mixtures were stored overnight at 4 °C. Precipitates were collected by centrifugation (15 min at 2000 *g*) and resuspended in one volume of demineralized water. After precipitation with two volumes of cold ethanol and centrifugation, pellets were dried at 55 °C. EPS was determined by measuring the dry weight or total carbohydrate content of the precipitates. The EPS sugar composition also was determined (see below). EPS slime, surrounding colonies grown on MRS/agar plates, was sampled with an inoculum loop and its sugar composition was determined (see below).

Determination of EPS monosaccharide composition

Complete hydrolysis of dried EPS was carried out by incubating samples for 2 h in 1 M H_2SO_4 at 100 °C. Monosaccharides were determined by high-performance anion-exchange chromatography using a Carbopac PA1 Column (4 × 250 mm, DIONEX) and pulsed amperometric detection. Sugars were eluted with a gradient of NaOH (0–90 mM in 25 min). Rhamnose (5 μg/ml), arabinose (50 μg/ml), galactose (30 μg/ml), glucose (105 μg/ml), xylose + mannose (26 + 51 μg/ml) and fructose (138 μg/ml) in 1 M H_2SO_4 were used as references. The detection limit for arabinose, rhamnose, galactose and glucose is 1 μg/ml, for xylose/mannose and fructose 5 μg/ml. Fructose in fructan of strain LB 121 was determined with the improved resorcinol reagent (Yaphe and Arsenault 1965).

EPS purification and analysis

EPS produced after 2 days growth on MRS-s or MRS-r was isolated as described above. Instead of drying, EPS was dialysed (cellulose dialysis tube, Sigma D-9777, cut-off 12 kDa) at 4 °C against water for 3 days, and then freeze-dried. EPS was redissolved in 0.1 M potassium phosphate (pH 6.7) and filtered over a 0.45 μm filter; 100 μl was used for high-performance gel-permeation chromatography (HP-GPC) analysis at room temperature using an isocratic HPLC pump (Waters model 501). Samples were injected with an automatic injector (Gilson model 231) on a Bio-Gel TSK guard column, followed by Bio-Gel TSK 60 and TSK 30 columns. Samples were eluted at a flow rate of 0.6 ml/min with 0.1 M potassium phosphate (pH 6.7) as mobile phase. EPS was detected with a refractive index detector (Erna ERC-7510).

Enzyme localization studies

Lactobacillus strains were grown in MRS medium with 30 g/l sucrose and harvested by centrifugation (15 min at 10 000 *g*) in the late exponential growth phase. Cells were washed twice with saline solution (8.5 g/l NaCl) and resuspended to the original volume in 0.05 M citric acid/0.10 M Na_2HPO_4 buffer, pH 5.5. Culture supernatants were dialysed (cellulose dialysis tube, Sigma D-9777, cut-off 12 kDa) for 24 h at 4 °C against the same citric acid/Na_2HPO_4 buffer (replaced four times by fresh buffer). The overall EPS biosynthetic enzyme activity was assayed by incubating washed cell suspensions and dialysed supernatants with sucrose (final concentration 33 g/l) for 0–24 h at 37 °C. EPS production was measured as the total carbohydrate content of ethanol-precipitable material.

Other assays

Protein was determined by the Lowry method (Lowry et al. 1951) using bovine serum albumin as standard. Cells were first boiled for 20 min in 1 M NaOH. Biomass (dry weight) was estimated by multiplying the protein content by a factor 2 (Gottschalk 1986). D- and L-lactic acid, and sucrose, were determined enzymatically using commercial kits (Boehringer Mannheim). The total amount of carbohydrate was determined with phenol/sulphuric acid (Dubois et al. 1956) with sucrose as standard. *Lactobacillus* strains were identified by polyacrylamide gel electrophoresis of proteins (van den Berg et al. 1993).

Results

Screening for EPS production

A total of 182 *Lactobacillus* strains were screened for EPS production on MRS-f, MRS-g, MRS-gal, MRS-l, MRS-m, MRS-r and MRS-s. Sucrose was an excellent substrate for abundant EPS synthesis; only a few strains produced EPS on other sugars. Sixty EPS-positive strains were identified; 17 strains produced more than 100 mg/l soluble EPS.

Monosaccharide composition of EPS

The EPS monosaccharide composition of the most productive strains, and of strains producing EPS from more than one sugar, was determined on liquid and solid modified MRS media. The main constituent of most EPS molecules was glucose and, to a lesser extent, fructose (Table 1). Other EPS monomers also were found occasionally, but only in small amounts. Strains LB 181 and LB 182 were exceptional, producing EPS on liquid MRS-s and MRS-l with not only glucose and

Table 1 Monosaccharide composition of partially purified extracellular polysaccharide (*EPS*) produced by *Lactobacillus* strains after 3 days incubation at 37 °C on liquid and/or solid modified MRS media with 100 g/l various sugars. *ND* not determined

Strain number	Sugar in medium	Solid/ liquid medium	Glucose in EPS % (w/w)	Fructose in EPS % (w/w)	Other sugars in EPS % (w/w)	Total amount of EPS (mg/l)
23	Sucrose	Liquid	84	0	16	255
	Sucrose	Solid	95	5	<1	ND
24	Sucrose	Liquid	85	0	15	130
33	Sucrose	Liquid	93	<1	7	>1000[a]
	Sucrose	Solid	96	4	<1	ND
34	Sucrose	Liquid	88	0	12	420
	Sucrose	Solid	98	1	<1	ND
44	Sucrose	Liquid	86	0	14	285
86	Sucrose	Liquid	92	2	6	1055
	Sucrose	Solid	92	5	3	ND
116	Sucrose	Liquid	95	1	4	1350
121	Sucrose	Liquid	24	75	1	4800
	Sucrose	Solid	65	34	1	ND
	Raffinose	Liquid	1	96	3	2655
180	Sucrose	Liquid	95	2	3	4050
181	Lactose	Liquid	25	0	75	160
	Sucrose	Liquid	21	30	49	145
182	Sucrose	Liquid	24	41	35	250
	Lactose	Liquid	29	0	71	110

[a] Separation of strain LB 33 biomass and EPS was inefficient, resulting in underestimation of the amount of EPS formed

fructose but also relatively high percentages of other sugars. On MRS-l, strain LB 181 produced EPS with 31.5% mannose, 25.5% glucose, 22% glucuronic acid, 17% galactose and 4% arabinose. On MRS-s, this strain produced EPS with 30% fructose, 23% mannose, 21% glucose, 17.5% glucuronic acid, 5% galactose and 3.5% arabinose. On MRS-l, strain LB 182 produced an EPS composed of 28.5% glucose, 27% mannose, 21% galactose, 19.5% glucuronic acid and 4% arabinose. On MRS-s, this strain produced EPS with 41% fructose, 24% glucose, 16% mannose, 11% glucuronic acid, 5% galactose and 3% arabinose.

Strain LB 121 was unusual in producing EPS with varying monosaccharide composition. The glucose and fructose ratio varied from 1:3 (liquid MRS-s) to 2:1 (solid MRS-s) and 1:96 (liquid MRS-r) (Table 1). These variations either reflect changes in sugar composition of a specific EPS, or synthesis of different EPS molecules (see below).

Growth and EPS production

Strains LB 121 and LB 180 were studied in more detail, in view of the high levels of EPS produced (by both) and the variations observed in EPS monosaccharide composition with growth conditions (strain LB 121). Both were identified as *Lactobacillus reuteri* strains by polyacrylamide gel electrophoresis of proteins (van den Berg et al. 1993).

Growth in MRS-s medium with increasing initial sucrose concentrations (0–100 g/l) resulted in increased biomass levels up to 20 g/l sucrose. EPS production by both strains, however, continued to increase in media containing up to 100 g/l sucrose, with strain LB 180 producing more than 20 g/l EPS (Fig. 1). Replacement of sucrose by equal amounts of glucose and fructose, supplied either as a mixture or separately, dramatically affected EPS production. Virtually no EPS was produced on MRS-g or MRS-(g + f) (< 0.1 g/l). The amount of biomass formed was about the same in MRS-s, MRS-g and MRS-(g + f). Relatively little

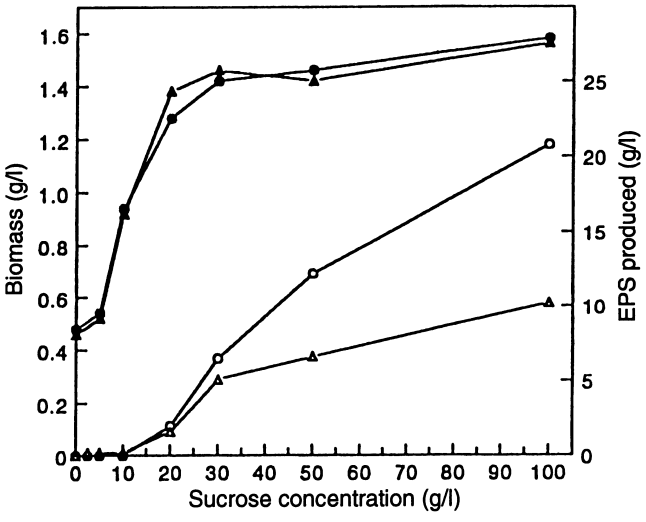

Fig. 1 Effects of the initial sucrose concentration on extracellular polysaccharide (*EPS*; ○ △) and biomass (● ▲) production by strains LB 121 and LB 180 after incubation under anaerobic conditions for 3 days at 37 °C in sucrose-containing MRS medium (MRS-s). The amount of EPS formed was quantified by measuring the total carbohydrate content of the ethanol precipitates. ○ ● LB 180; △ ▲ LB 121

growth (0.15–0.20 g/l biomass) and virtually no EPS production (< 0.1 g/l) occurred in MRS-f medium.

Anaerobic growth in MRS-s medium without pH control resulted in abundant EPS production (over 10 g/l) by both strains (Figs 2, 3). Several differences were observed between the two cultures. Strain LB 121 continued to grow and to produce lactate for 50 h. Growth of strain LB 180 (and lactate production) terminated after 20 h, reaching relatively low final lactate levels. Strains LB 121 and LB 180 both produced EPS in the early exponential growth phase, but only strain LB 180 continued to produce EPS in the stationary phase. Strain LB 121 rapidly produced high levels of EPS, reaching a maximum concentration after 15 h. Strain LB 180 produced EPS less rapidly but converted relatively more sucrose into EPS and less into lactate. Growth, EPS synthesis and sucrose utilization of both cultures ceased before exhaustion of sucrose from the medium.

Growth of both strains in MRS-s medium was also studied in batch fermenters with automatic pH control. At pH 5.8, both strains completely consumed the sucrose from the medium within 30 h (Figs 4C, 5C), resulting in a much higher lactate production (Figs 4D, 5D) than in the absence of pH control (Figs 2B, 3B). Control of the pH at 4.8 resulted in reduced rates of sucrose consumption and lactate production by both strains (Figs 4C, D and 5C, D). Both strains formed much more biomass at pH 5.8 (Figs 4A, 5A), but the total amount of EPS produced after 30 h of fermentation was almost the same at both pH values for both strains (Figs 4B, 5B).

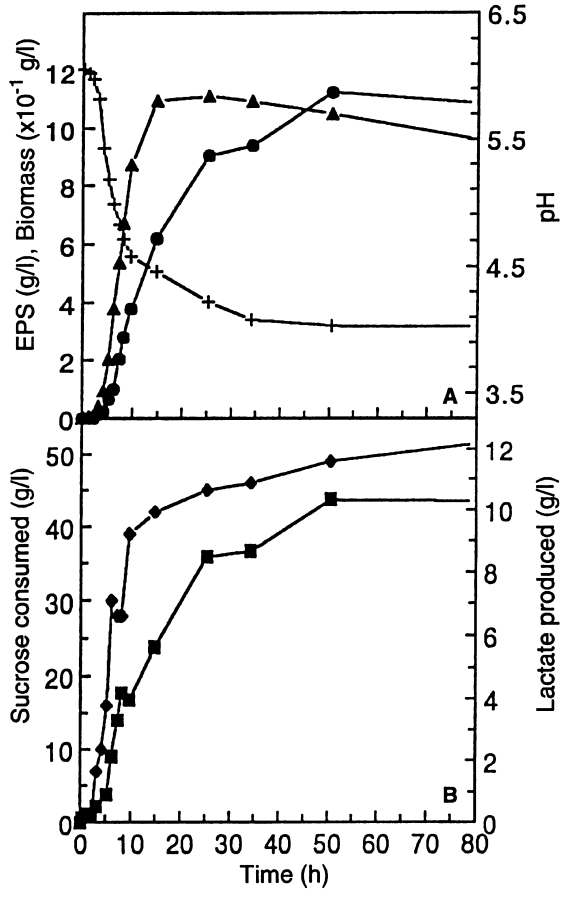

Fig. 2A, B Growth and EPS production of LB 121 under anaerobic conditions at 37 °C in MRS-s medium without pH control. **A** ● Biomass, ▲ EPS production, + pH; **B** ◆ sucrose consumption, ■ lactate production

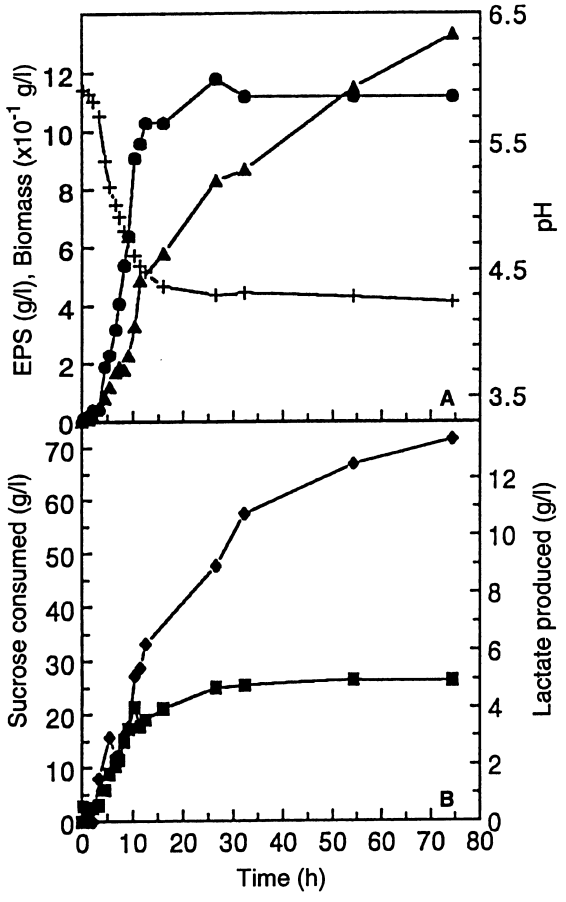

Fig. 3A, B Growth and EPS production of LB 180 under anaerobic conditions at 37 °C in MRS-s medium without pH control. **A** ● biomass, ▲ EPS production, + pH; **B** ◆ sucrose consumption, ■ lactate production

Localization of EPS biosynthetic enzymes

Washed cell suspensions and dialysed culture supernatants of strain LB 121 converted sucrose into EPS (Table 2). EPS biosynthetic enzyme(s) of LB 121 thus occur both in a cell-free and a cell-associated form. EPS production by strain LB 180 was much higher in culture supernatants than in cell suspensions. EPS biosynthetic enzyme(s) of strain LB 180 thus occur(s) largely in a cell-free form. With both strains there were no indications of cell lysis.

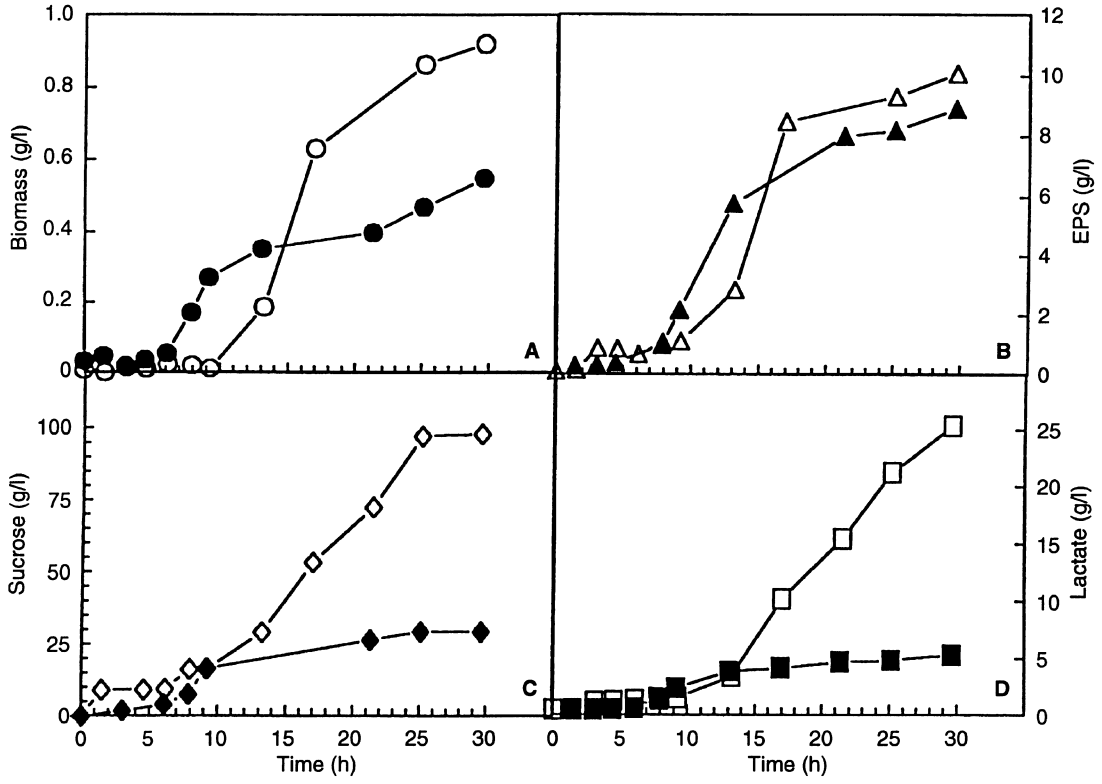

Fig. 4A–D Growth of strain LB 121 under anaerobic conditions at 37 °C in MRS-s medium in fermentors with automatic pH control at pH 5.8 (○ △ ◇ □) or pH 4.8 (● ▲ ◆ ■). **A** Biomass, **B** EPS production, **C** sucrose consumption, **D** lactate production

EPS analysis

HP-GPC analysis of purified EPS of strain LB 121 grown on MRS-s showed two peaks with retention times of 20 min and 27 min. EPS of cells grown on MRS-r only showed a peak at 27 min. Also EPS produced by strain LB 180 grown on MRS-s only showed a single peak at 20 min. Strain LB 180 did not produce EPS on MRS-r.

Monosaccharide analysis of the HP-GPC peak fractions revealed that strain LB 121 synthesized two polymers on sucrose, a glucan (retention time 20 min) and a fructan (retention time 27 min); on raffinose only the fructan was produced (retention time 27 min). Strain LB 180 produced only a glucan on MRS-s (retention time 20 min).

Discussion

Screening for EPS synthesis by lactic acid bacteria is usually carried out on agar media with relatively low glucose or lactose concentrations (van den Berg et al. 1993; Cerning 1990; Vedamuthu and Neville 1986; Vescovo et al. 1989). Following this approach, EPS producers are identified on the basis of colony ropiness. Our data show that growth in liquid MRS medium with relatively high sugar concentrations provides an excellent alternative, yielding a large number of EPS-positive strains (60 out of 182 strains tested). In comparison, use of skimmed-milk medium with 10 g/l glucose only yielded 30 EPS-producing strains out of 607 strains tested (van den Berg et al. 1993). The new method presented in this paper, using high sugar concentrations in liquid media, is more laborious but allows identification of strains with a high EPS production potential, and does not depend on EPS ropiness. Several (10%) strains produced over 100 mg/l EPS; 5 strains produced more than 1 g/l EPS (Table 1), levels that are 10- to 100-fold higher than those previously reported for lactobacilli (van den Berg et al. 1995; Grobben et al. 1995).

EPS molecules produced in relatively large amounts (>100 mg/l) contained almost exclusively glucose (and fructose) (Table 1). This was not due to the screening method used: several strains produced relatively low amounts of EPS with other monosaccharides (mannose, arabinose, galactose). Sucrose clearly was the best substrate for EPS synthesis (Table 1) and appears to be the direct substrate for EPS synthesis by extracellular enzymes in strains LB 121 and LB 180 (Table 2). Synthesis of glucans (and fructans) by strains LB 121 and LB 180, and most of the other high-EPS-producing strains identified (Table 1), thus may involve sucrase-type extracellular (Table 2) enzymes (Dols et al. 1997; Monchois et al. 1997; Robyt 1995; Robyt and Walseth 1978). These glycosyltransferase enzymes catalyse transfer of glucosyl or fructosyl residues from sucrose to a glucan or a fructan polymer respectively. Low-EPS-producing strains (i.e. LB 181, LB 182), on the other hand, syn-

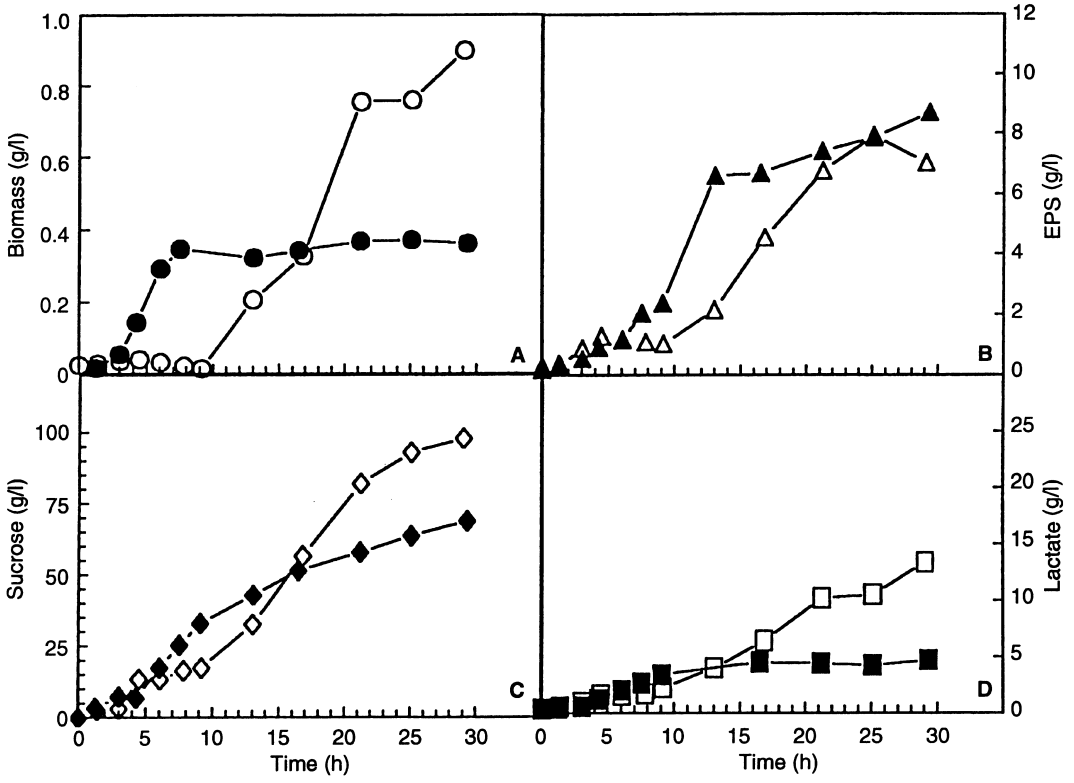

Fig. 5A–D Growth of strain LB 180 under anaerobic conditions at 37 °C in MRS-s medium in fermentors with automatic pH control at pH 5.8 (○ △ ◇ □) or pH 4.8 (● ▲ ◆ ■). **A** Biomass, **B** EPS production, **C** sucrose consumption, **D** lactate production

thesize heteropolysaccharides and may employ intracellular nucleotide-sugar-dependent glycosyltransferases with different sugar substrate specificity (van den Berg et al. 1995; van Kranenburg et al. 1997; Stingele et al. 1996).

The EPS monosaccharide composition of strain LB 121 varied with the growth conditions (liquid or solid medium) and sugar substrates (sucrose or raffinose) supplied in the medium (Table 1). These differences may be caused by variations in expression or stability of the glucan and fructan biosynthetic enzymes.

Table 2 EPS production by washed cell suspensions and dialysed supernatants of (*sup.*) of LB 121 and LB 180, obtained from cultures grown in MRS medium with 30 g/l sucrose. Incubations were under anaerobic conditions at 37 °C, pH 5.5 with 33 g/l sucrose. EPS was determined by measuring the total carbohydrate content of ethanol precipitates

Incubation time (h)	EPS produced (g/l)			
	LB 121 cells	LB 121 sup.	LB 180 cells	LB 180 sup.
0	0	0	0	0
0.5	0.38	0.72	<0.1	0.54
1	0.89	1.1	<0.1	0.80
2	1.5	1.6	<0.1	1.1
4	2.4	2.3	0.11	1.1
24	5.2	5.2	0.48	1.6

Moreover, raffinose is known to be a substrate for levansucrase, but not for glucansucrase (Robyt and Walseth 1978). This explains the observed synthesis of only the fructan by LB 121 on raffinose medium (Table 1). Also *Streptococcus mutans* strain Ingbritt (Russell 1978) produces a water-soluble EPS consisting of a dextran (40%) and a fructan (60%) from sucrose. With raffinose, the only product was a water-soluble fructan.

Glucan and fructan synthesis by lactic acid bacteria (*Leuconostoc* and *Streptococcus* species) is well documented (Monchois et al. 1997; Robyt 1995; Robyt and Walseth 1978; Russell 1978). Only a few reports have described glucan synthesis by a few *Lactobacillus* species (Dunican and Seeley 1965; Pidoux et al. 1990; Sharpe et al. 1972). Our data show that the ability to produce glucans is, in fact, widespread in the genus *Lactobacillus*. This is the first report of fructan production by a *Lactobacillus* species.

Both LB 121 and LB 180 were identified as strains of *Lactobacillus reuteri*, but their pattern of EPS synthesis in MRS-s medium without pH control differed (Figs 2, 3) from being strictly growth-associated (strain LB 121) to continued synthesis after growth had stopped (LB 180). In pH-controlled batch fermentors both strains produced approximately the same amount of EPS at pH 4.8 and pH 5.8 (t = 30 h), although at pH 4.8 less biomass and lactate were formed, and less sucrose was consumed (Figs 4, 5). Not all of the sucrose consumed was recovered as EPS and lactate (plus equimolar amounts of ethanol and CO_2): the action of sucrase-type enzymes will not only result in EPS syn-

thesis but also in the formation of glucose and fructose, part of which subsequently will be metabolized again by the cells. Also short EPS fragments (oligosaccharides) may have been produced that were not precipitated with 67% ethanol.

In conclusion, we have used a new method for screening lactic acid bacteria for EPS production that did not depend on EPS ropiness, using high sugar concentrations in liquid growth medium. This allowed identification of *Lactobacillus* strains producing large amounts of EPS. The EPS molecules produced by strains LB 121 and LB 180 may possess interesting properties for industrial applications. EPS structures, properties of the sucrase-type of enzymes, and factors determining EPS monosaccharide composition and yields are currently studied in more detail.

Acknowledgements We thank C. Verbeek (TNO Nutrition and Food Research, Department of Biochemistry and Physical Chemistry) for technical assistance with the monosaccharide analyses and F. Kingma (NIZO, Department of Microbiology) for technical assistance with HP-GPC analyses. The research described has been (partly) funded by the Programma Bedrijfsgerichte Technologie Stimulering (PBTS) of the Dutch Ministry of Economic Affairs and by the Investment and Development Company for the North of the Netherlands (NOM).

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